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Intracellular Cannabinoid Type 1 (CB_1) Receptors Are Activated by Anandamide*[S]

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Recent studies have demonstrated that the majority of endogenous cannabinoid type 1 (CB_1) receptors do not reach the cell surface but are instead associated with endosomal and lysosomal compartments. Using calcium imaging and intracellular microinjection in CB_1 receptor-transfected HEK293 cells and NG108-15 neuroblastoma × glioma cells, we provide evidence that anandamide acting on CB_1 receptors increases intracellular calcium concentration when administered intracellularly but not extracellularly. The calcium-mobilizing effect of intracellular anandamide was dose-dependent and abolished by pretreatment with SR141716A, a CB_1 receptor antagonist. The anandamide-induced calcium increase was reduced by blocking nicotinic acid-adenine dinucleotide phosphate- or inositol 1,4,5-trisphosphate-dependent calcium release and abolished when both lysosomal and endoplasmic reticulum calcium release pathways were blocked. Taken together, our results indicate that, in CB_1 receptor-transfected HEK293 cells, intracellular CB_1 receptors are functional; they are located in acid-filled calcium stores (endolysosomes). Activation of intracellular CB_1 receptors releases calcium from endoplasmic reticulum and lysosomal calcium stores. In addition, our results support a novel role for nicotinic acid-adenine dinucleotide phosphate in cannabinoid-induced calcium signaling.

Although most studies on the cannabinoid type 1 (CB_1)[3] receptor have focused on its localization to the plasma membrane, increasing evidence suggests that not only are CB_1 receptors highly expressed in intracellular compartments but also that these intracellular receptors are functional. An early study in N18TG2 neuroblastoma cells demonstrated localization of CB_1 receptors to nuclear membranes in cells not treated with agonists (1). More recent studies have suggested that the CB_1 receptor undergoes constitutive endocytosis in both neurons and transfected cells (2–4). Rozenfeld and Devi (5) found that the majority of the endogenous CB_1 receptors do not reach the cell surface but are instead associated with endosomal and lysosomal compartments. They further demonstrated that, during trafficking, the lysosomal CB_1 receptors may interact with $G\alpha_i$ proteins. Another recent report indicated that the intracellular pool of CB_1 receptors does not contribute to cell-surface repopulation (6), and thus, it may have a distinct yet unknown function from the membrane CB_1 receptors.

Most previous studies examining cannabinoids, including anandamide, have demonstrated a nonspecific effect in that non-transfected cells also showed a calcium response (7, 8). One study showed that only the indole derivative WIN55,212-2 was able to increase intracellular calcium via CB_1 receptor coupling to $G_{q/11}$ proteins (9). Many studies have shown that cannabinoids can inhibit a voltage-sensitive calcium channel present in neurons and neuroblastoma cells (*e.g.* Refs. 7, 10, and 11). In this study, we show that anandamide increases intracellular calcium concentration but only when injected in CB_1 receptor-transfected HEK293 cells and NG108-15 neuroblastoma × glioma cells and provide evidence that intracellular CB_1 receptors are functional.

EXPERIMENTAL PROCEDURES

Cell Culture—CB_1 receptor-transfected HEK293 cells have been described previously (12). Briefly, stably transfected HEK293 cell lines were created by transfection with human CB_1-pcDNA3 using Lipofectamine reagent (Invitrogen) and selected in DMEM containing G418 (Geneticin; 1 mg/ml) and 10% fetal calf serum at 37 °C in a 5% CO_2 incubator. Colonies of ~500 cells were picked (~2 weeks post-transfection) and allowed to expand and then were tested for expression of receptor by immunostaining. Cell lines containing moderate-to-high levels of receptor expression were tested for receptor-binding properties. The cell line was maintained in DMEM and 10% fetal calf serum with 0.7 mg/ml G418 at 37 °C in a 5% CO_2 incubator. NG108-15 cells (mouse neuroblastoma × rat glioma) were cultured in DMEM without sodium pyruvate, 10% fetal bovine serum, 0.1 mM hypoxanthine, 400 nM aminopterin, and 0.016% thymidine as suggested by the manufacturer (American Type Culture Collection, Manassas, VA).

Calcium Imaging—Intracellular Ca^{2+} measurements were performed as described previously (13, 14). Briefly, cells were incubated with 5 μM fura-2/AM (Invitrogen) in Hanks' balanced salt solution at room temperature for 45 min in the dark, washed three times with dye-free buffer, and incubated for another 45 min to allow for complete de-esterification of the dye. Coverslips (25-mm diameter) were subsequently mounted

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[3] The abbreviations used are: CB_1, cannabinoid type 1; NAADP, nicotinic acid-adenine dinucleotide phosphate; IP_3, inositol 1,4,5-trisphosphate; PLC, phospholipase C.

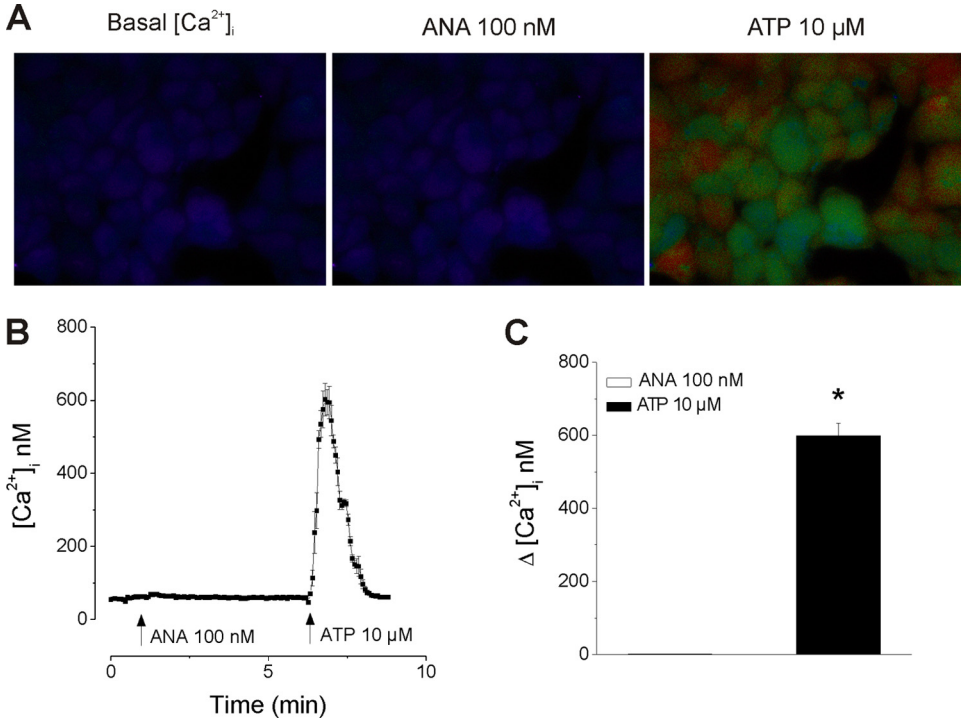

FIGURE 1. **Extracellular administration of ATP but not anandamide increases $[Ca^{2+}]_i$ in CB_1 receptor-transfected HEK293 cells.** *A*, fluorescent images of fura-2/AM-loaded CB_1 receptor-transfected HEK293 cells illustrating the levels of basal $[Ca^{2+}]_i$ (*left panel*) and during administration of anandamide (*ANA*; 100 nM; *middle panel*) or ATP (10 μM; *right panel*). Low levels of $[Ca^{2+}]_i$ are seen as *blue* fluorescence and higher levels as *green* and *red* fluorescence. *B*, averaged Ca^{2+} responses to anandamide (*first arrow*) or ATP (*second arrow*) from $n = 46$ cells. *C*, comparison of the amplitude of Ca^{2+} responses produced by extracellular administration of anandamide or ATP. *, $p < 0.05$ compared with basal $[Ca^{2+}]_i$.

in an open bath chamber (RP-40LP, Warner Instruments, Hamden, CT) on the stage of an inverted microscope (Nikon Eclipse TiE, Optical Apparatus Co., Ardmore, PA). The microscope was equipped with a Perfect Focus System and a Photometrics CoolSNAP *HQ*^{2} CCD camera (Roper Scientific and Optical Apparatus Co.). During the experiments, the Perfect Focus System was activated. Fura-2/AM fluorescence (emission = 510 nm) following alternate excitation at 340 and 380 nm was acquired at a frequency of 0.25 Hz. Images were acquired and analyzed using Nikon NIS-Elements AR 3.1 software (Optical Apparatus Co.). The ratio of the fluorescence signals (340/380 nm) was converted to Ca^{2+} concentrations (15). In Ca^{2+}-free experiments, $CaCl_2$ was omitted, and 2.5 mM EGTA was added.

Intracellular Microinjection—Injections were performed using Femtotips II, InjectMan NI 2, and FemtoJet systems (Eppendorf) as described previously (13, 14). Pipettes were back-filled with an intracellular solution composed of 110 mM KCl, 10 mM NaCl, and 20 mM HEPES (pH 7.2) or the drugs to be studied. The injection time was 0.4 s at 60 hectopascals with a compensation pressure of 20 hectopascals to ensure that <0.1% of the cell volume was microinjected.

Immunocytochemistry—HEK293 cells were transfected with CB_1-GFP cDNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were fixed in 4% paraformaldehyde for 20 min at room temperature and processed for LAMP-1 (lysosome-associated membrane protein 1) (16) immunoreactivity. Cells were washed three times with PBS and then permeabilized with 0.5% Triton X-100 in PBS for 10 min. Cells were blocked with 3% BSA in PBS for 30 min and incubated with LAMP-1 (mouse IgG, 1:500 dilution; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) for 2 h at room temperature. Cells were washed three times with PBS and then incubated with Alexa 568-labeled goat anti-mouse antibodies (1:2000 dilution; Molecular Probes, Eugene, OR) for 1 h. After washing three times with PBS, cells were mounted with DAPI Fluoromount G (Southern-Biotech, Birmingham, AL) and examined under a confocal laser scanning microscope (Leica TCS SP5) with excitation wavelengths set to 405 nm for DAPI, 488 nm for GFP, and 561 nm for Alexa 568 in the sequential mode.

RESULTS

Intracellular but Not Extracellular Administration of Anandamide Increases Cytosolic Ca^{2+} Concentration in Cells Expressing CB_1 Receptors—HEK293 cells stably expressing the human CB_1 receptor (12) were examined for their responses to anandamide; non-transfected HEK293 cells served as a control. We also tested the responses to anandamide in NG108-15 cells, which endogenously express CB_1 but not CB_2 receptors (17). The basal cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was 65 ± 0.6 nM ($n = 124$) in CB_1 receptor-expressing HEK293 cells, 66 ± 0.8 nM ($n = 75$) in non-transfected HEK293 cells, and 64 ± 0.7 nM ($n = 84$) in NG108-15 cells. Extracellular administration of anandamide did not produce a significant increase in $[Ca^{2+}]_i$, whereas the cells responded to ATP (10 μM) administration by an increase in $[Ca^{2+}]_i$ of 599 ± 34 nM ($n = 46$) (Fig. 1). The response to ATP was used as proof of integrity of G_q-dependent pathways in these cells, as HEK293 cells endogenously express P2Y receptors (18). Similar results were obtained when anand-

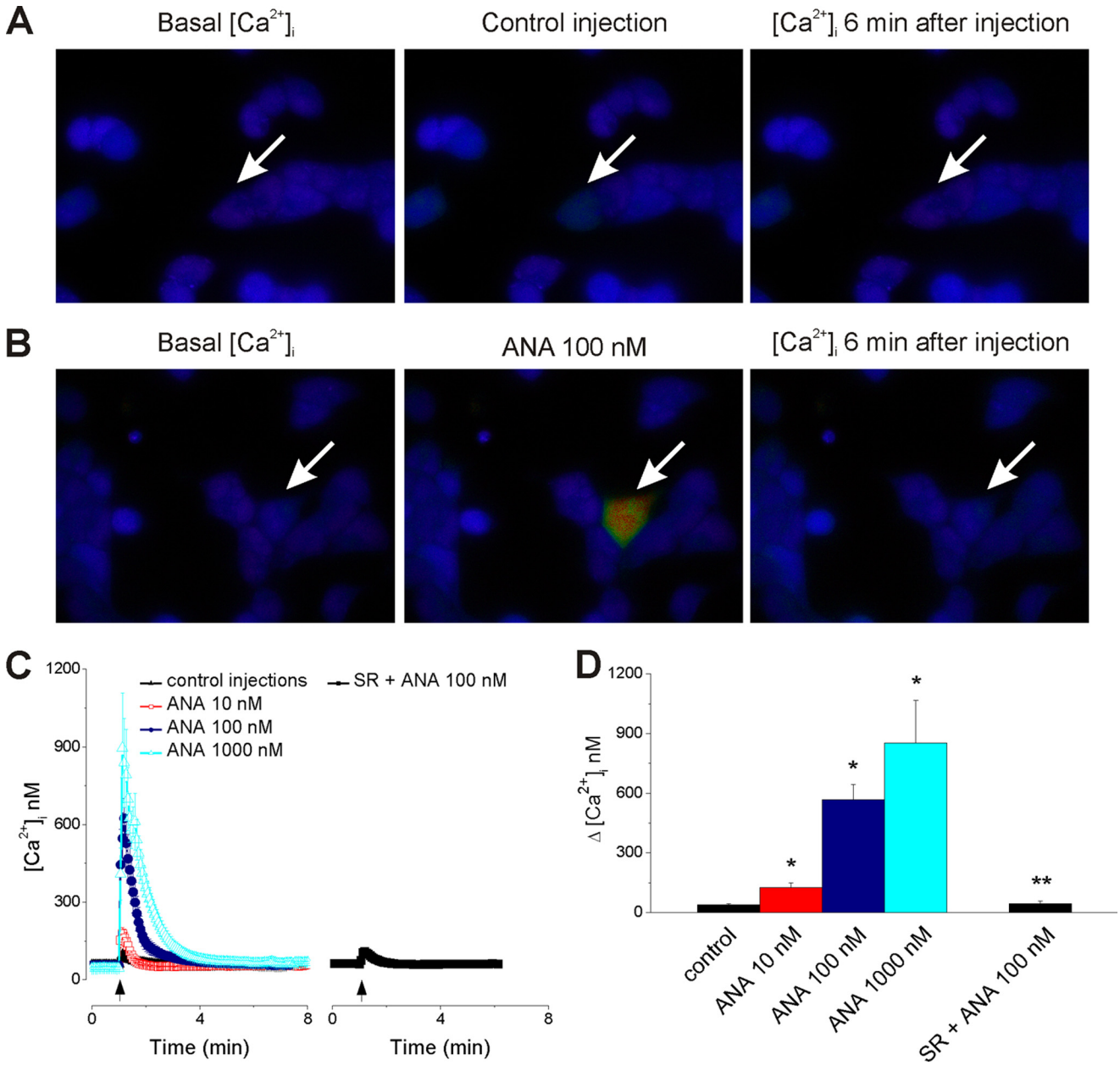

FIGURE 2. **Intracellular administration of anandamide increases $[Ca^{2+}]_i$ in CB_1 receptor-expressing HEK293 cells.** *A* and *B*, fluorescent images of fura-2/AM-loaded CB_1 receptor-transfected HEK293 cells illustrating the levels of $[Ca^{2+}]_i$ before injection (*left panels*), during control or anandamide (*ANA*; 100 nM) injection (*middle panels*), and 6 min after injection (*right panels*). Low levels of $[Ca^{2+}]_i$ are seen as *blue* fluorescence and higher levels as *green* and *red* fluorescence. The *arrows* indicate the injected cells. *C*, intracellular administration of anandamide (10, 100, and 1000 nM) produced a fast, robust, and transitory increase in $[Ca^{2+}]_i$ that was concentration-dependent. Averaged traces from six experiments are shown. Pretreatment with the CB_1 receptor antagonist SR141716A (*SR*) abolished the response to anandamide. *D*, comparison of the increases in $[Ca^{2+}]_i$ produced by different concentrations (10–1000 nM) of anandamide in the absence or presence of the antagonist. *, $p < 0.05$ compared with the control; **, $p < 0.05$ compared with anandamide (100 nM).

amide was extracellularly administered to non-transfected HEK293 cells (supplemental Fig. 1).

Anandamide (10, 100, and 1000 nM) administered by intracellular injection produced concentration-dependent increases in $[Ca^{2+}]_i$ of 126 ± 22, 567 ± 76, and 854 ± 212 nM, respectively ($n = 6$ for each concentration tested). The Ca^{2+} response to intracellular administration of anandamide was fast, robust, and transitory, similar to that induced by the injection of second messengers (see Fig. 5). Fig. 2 shows examples of representative experiments (*A* and *B*) and the dose-response relationship (*C* and *D*). The effect was CB_1 receptor-specific, as pretreatment with SR141716A (1 μM, 15 min), a CB_1 receptor antagonist (19), prevented the anandamide-induced increase in $[Ca^{2+}]_i$ (Fig. 2, *C* and *D*). Moreover, intracellular injection of anandamide in non-transfected HEK293 cells did not produce an increase in $[Ca^{2+}]_i$ higher than the control injection (supplemental Fig. 2).

Functional CB_1 Receptors Are Localized on Lysosomes—The increase in $[Ca^{2+}]_i$ produced by intracellular administration of anandamide was markedly reduced by pretreatment with bafilomycin A1 (1 μM, 1 h), a blocker of lysosomal Ca^{2+} release, but was not affected by pretreatment with brefeldin A (10 μM, 1 h),

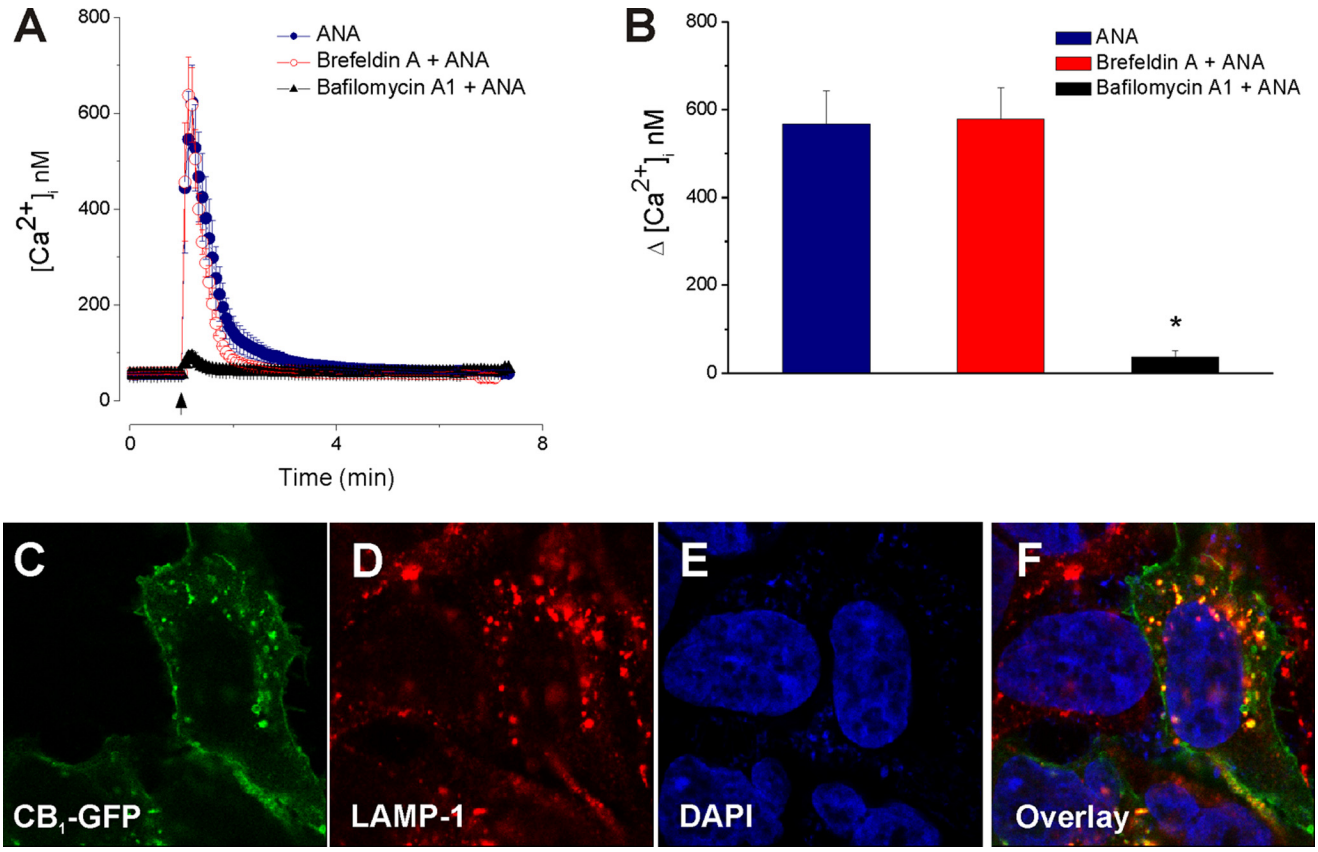

FIGURE 3. **Functional CB_1 receptors are located in lysosomes.** *A*, pretreatment with bafilomycin A1 (1 μM, 1 h; ▲) but not with brefeldin A (10 μM, 1 h; ○) prevented the anandamide (*ANA*)-induced Ca^{2+} increase (●). *B*, comparison of the increases in $[Ca^{2+}]_i$ produced by the intracellular injection of anandamide in cells without pretreatment (*first bar*) or pretreated with brefeldin A (*second bar*) or bafilomycin A1 (*third bar*). *, $p < 0.05$ compared with anandamide (100 nM). *C–F*, confocal images of CB_1-GFP-expressing HEK293 cells (*C*). The lysosomes were labeled with LAMP-1-Alexa 568 (*D*), and the nucleus was labeled with DAPI (*E*). In the overlay image (*F*), the colocalization of intracellular CB_1 receptors with lysosomes is seen as *orange* fluorescence.

which disrupts the Golgi apparatus (Fig. 3, *A* and *B*). In cells pretreated with brefeldin A, anandamide (100 nM) increased $[Ca^{2+}]_i$ by 579 ± 71 nM ($n = 6$), whereas pretreatment with bafilomycin A1 markedly reduced the response to anandamide to 37 ± 14 nM (Fig. 3*B*). Bafilomycin A1, a V-type ATPase inhibitor (20), blocks the release of Ca^{2+} from lysosome-like acidic stores in a variety of other cell types (21–25). In CB_1-GFP-transfected HEK293 cells, in which lysosomes were labeled with LAMP-1 (16), intracellular CB_1 receptors were colocalized with lysosomes (Fig. 3, *C–F*).

CB_1 Receptor-expressing HEK293 Cells Have Functional Internal Calcium Stores—In Ca^{2+}-free EGTA (2.5 mM)-containing Hanks' balanced salt solution, thapsigargin (1 μM), a sarcoplasmic/endoplasmic reticulum ATPase inhibitor, increased $[Ca^{2+}]_i$ (Fig. 4). Adding monensin (50 μM) on the plateau of the thapsigargin-induced response produced a supplemental increase in $[Ca^{2+}]_i$ (Fig. 4). These results indicate that HEK293 cells stably transfected with the CB_1 receptor possess both endoplasmic reticulum and lysosomal Ca^{2+} stores.

Nicotinic acid-adenine dinucleotide phosphate (NAADP) is a second messenger that releases Ca^{2+} from lysosomes (26), whereas inositol 1,4,5-trisphosphate (IP_3) releases Ca^{2+} from the endoplasmic reticulum via IP_3 receptor activation (27). Intracellular administration of NAADP (50 nM) produced a fast and transitory increase in $[Ca^{2+}]_i$ of 296 ± 54 nM ($n = 6$) that was prevented by pretreatment with Ned-19

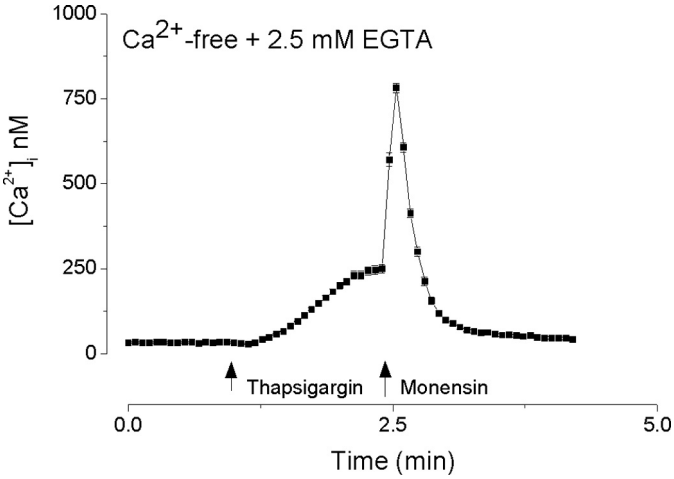

FIGURE 4. **Evaluation of endoplasmic reticulum and lysosomal Ca^{2+} stores in HEK293 cells.** HEK293 cells have both thapsigargin-sensitive and monensin-sensitive internal Ca^{2+} stores. Administration of thapsigargin (*first arrow*) or monensin (*second arrow*) increased $[Ca^{2+}]_i$, indicating the presence of endoplasmic reticulum and lysosomal Ca^{2+} stores in CB_1 receptor-transfected HEK293 cells.

(5 μM, 15 min), an inhibitor of NAADP signaling (28). Intracellular injection of IP_3 (50 nM) increased $[Ca^{2+}]_i$ by 511 ± 41 nM; co-administration of IP_3 and heparin (100 μg/ml) in cells pretreated with xestospongin C (10 μM, 15 min) did not increase $[Ca^{2+}]_i$ (Fig. 5).

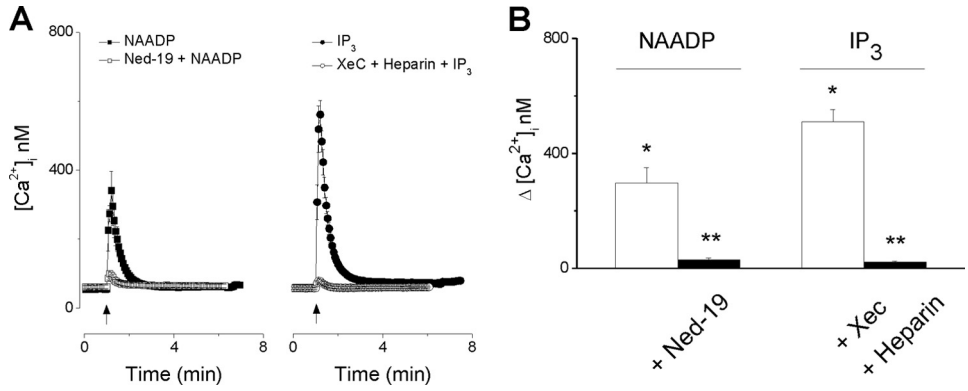

FIGURE 5. **NAADP- and IP_3-dependent Ca^{2+} release in HEK293 cells.** *A*, intracellular administration of Ca^{2+}-mobilizing second messengers NAADP (50 nM) and IP_3 (50 nM) produced a fast, robust, and transitory increase in $[Ca^{2+}]_i$, which was prevented by pretreatment with the antagonists Ned-19 and xestospongin C (*XeC*) and heparin, respectively. *B*, comparison of the amplitude of $[Ca^{2+}]_i$ increases produced by NAADP and IP_3 in the absence or presence of their specific antagonists. *, $p < 0.05$ compared with the control; **, $p < 0.05$ compared with the corresponding agonist.

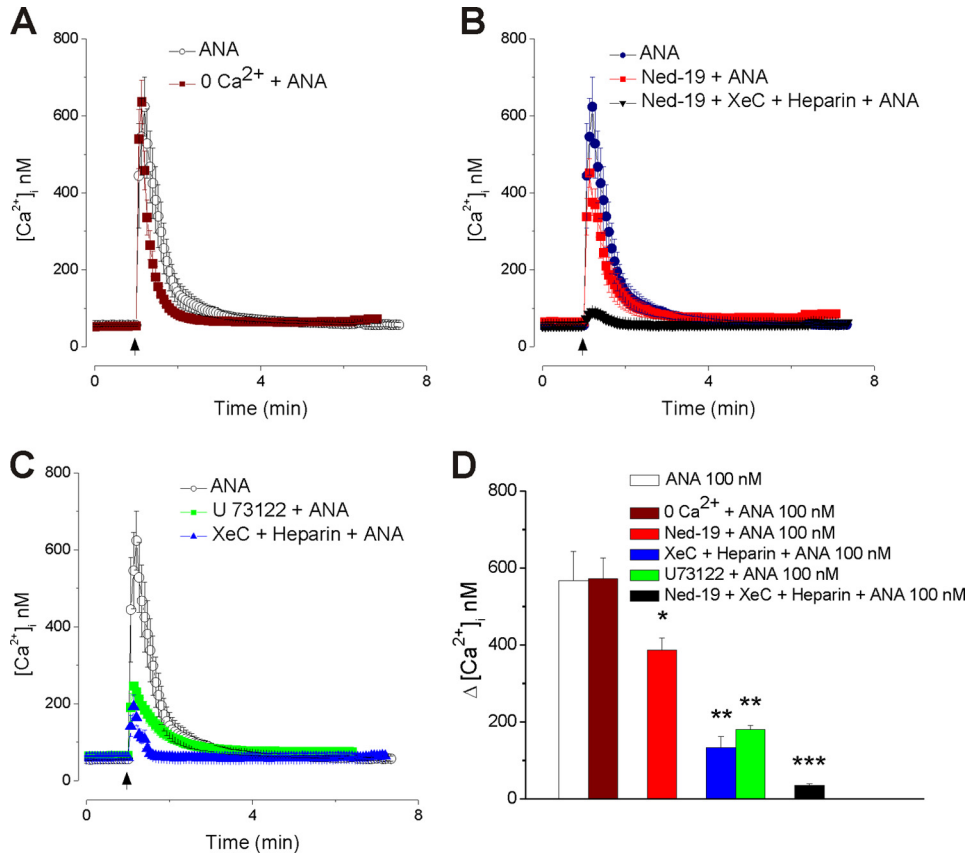

FIGURE 6. **Activation of CB_1 receptor by anandamide involves NAADP and IP_3-dependent Ca^{2+} pathways.** *A–C*, averaged Ca^{2+} responses ($n = 6$) to intracellular injection of anandamide (*ANA*) in Ca^{2+}-free saline (*A*) and in the presence of the indicated antagonists of the NAADP-dependent (*B*) and IP_3-dependent (*C*) Ca^{2+} pathways. The *arrows* indicate the injection of anandamide. The response to anandamide was reduced by blocking NAADP signaling with Ned-19, IP_3 receptor antagonists (xestospongin C (*XeC*) and heparin), and a PLC inhibitor (U73122) and abolished by blocking both NAADP- and IP_3-dependent Ca^{2+} release. *D*, comparison of the amplitude of the $[Ca^{2+}]_i$ increases produced by anandamide in regular Ca^{2+} and Ca^{2+}-free saline and in the presence of the indicated antagonists. *, $p < 0.05$ compared with anandamide (100 nM); **, $p < 0.05$ compared with anandamide and Ned-19 + anandamide; ***, $p < 0.05$ compared with anandamide + xestospongin C + heparin or with U73122 + anandamide.

Activation of the CB_1 Receptor Releases Ca^{2+} from Lysosomes and the Endoplasmic Reticulum—In Ca^{2+}-free saline, intracellular injection of anandamide (100 nM) increased $[Ca^{2+}]_i$ by 572 ± 55 nM ($n = 6$; *versus* by 567 ± 76 nM in regular Ca^{2+}-containing Hanks' balanced salt solution). In cells pretreated with Ned-19 (5 μM, 15 min), intracellular injection of anandamide (100 nM) produced an increase in $[Ca^{2+}]_i$ of 387 ± 31 nM ($n = 6$), which was lower than that produced by anandamide in untreated cells (567 ± 76 nM) (Fig. 6, *B* and *D*). The response to anandamide was reduced by blocking IP_3 receptors with xestospongin C and heparin (133 ± 29 nM, $n = 6$) (Fig. 6*C*) and abolished when the Ca^{2+} release from both NAADP- and IP_3-sensitive Ca^{2+} stores was blocked (Fig. 6, *C* and *D*). Because the amplitude of the Ca^{2+} response to anandamide was higher than that accounted for by the NAADP-dependent and IP_3-dependent Ca^{2+} release together, a Ca^{2+}-induced Ca^{2+} release

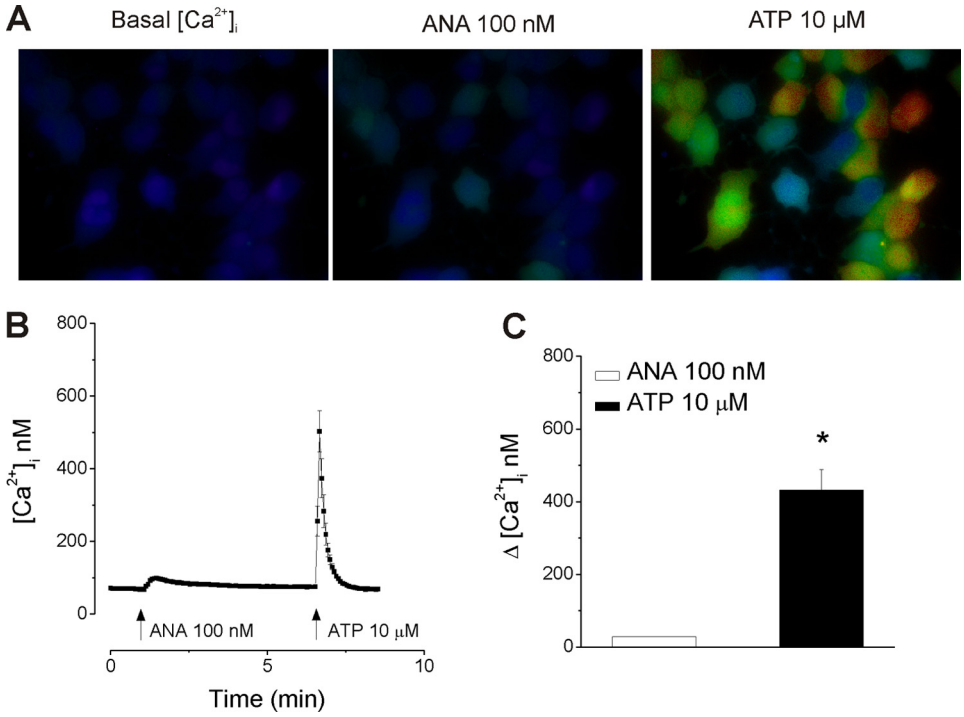

FIGURE 7. **Extracellular administration of ATP but not anandamide increases $[Ca^{2+}]_i$ in NG108-15 cells.** *A*, fluorescent images of fura-2/AM-loaded NG108-15 cells, which endogenously express CB_1 receptors, illustrating the levels of basal $[Ca^{2+}]_i$ (*left panel*) and during administration of anandamide (*ANA*; 100 nM; *middle panel*) or ATP (10 μM; *right panel*). Low levels of $[Ca^{2+}]_i$ are seen as *blue* fluorescence and higher levels as *green* and *red* fluorescence. *B*, averaged Ca^{2+} responses to anandamide (*first arrow*) or ATP (*second arrow*) from $n = 52$ cells. *C*, comparison of the amplitude of Ca^{2+} responses produced by extracellular administration of anandamide or ATP. *, $p < 0.05$ compared with basal $[Ca^{2+}]_i$.

mechanism is likely responsible for the difference. In addition, pretreatment with U73122, a phospholipase C (PLC) inhibitor (29), reduced but did not abolish the Ca^{2+} response to anandamide (Fig. 6*C*).

Next, to validate our results obtained in CB_1 receptor-transfected cells in endogenously expressing cells, we carried out calcium imaging experiments in NG108-15 cells, which endogenously express CB_1 but not CB_2 receptors (17). In NG108-15 cells, extracellular administration of anandamide did not produce a significant increase in $[Ca^{2+}]_i$, whereas the administration of ATP (10 μM) increased $[Ca^{2+}]_i$ by 432 ± 57 nM ($n = 52$) (Fig. 7).

In NG108-15 cells, intracellular injection of anandamide (100 nM) produced a fast, robust, and transitory increase in $[Ca^{2+}]_i$ of 356 ± 30 nM ($n = 6$). Examples of representative experiments are shown in Fig. 8 (*A–C*). Pretreatment with the CB_1 receptor antagonist SR141716A (1 μM, 15 min) (19) markedly decreased the response to anandamide (Fig. 8, *C* and *D*), supporting a CB_1 receptor-specific effect.

DISCUSSION

Endocannabinoids are lipid messengers generated intracellularly; they cannot be stored in vesicles (30, 31). As a result, they may target intracellular or extracellular cannabinoid receptors from the same cells or in neighboring cells. On the basis of the fact that other G protein-coupled receptors such as those for angiotensin II (32, 33) are active when stimulated from inside the cells, we tested whether or not CB_1 receptors can be activated intracellularly. We addressed this question in cells stably transfected with or endogenously expressing CB_1 receptors by monitoring the $[Ca^{2+}]_i$ of fura-2/AM-loaded cells in response to extracellular and intracellular administration of anandamide.

Although extracellular administration of anandamide did not enhance $[Ca^{2+}]_i$, intracellular injection of anandamide elicited a dose-dependent increase in $[Ca^{2+}]_i$, supporting a functional intracellular location of CB_1 receptors. The anandamide-induced $[Ca^{2+}]_i$ increase was absent after pretreatment with a CB_1 receptor antagonist or in cells lacking the CB_1 receptor (non-transfected HEK293 cells), further supporting the specificity of the response.

The next series of experiments were designed to characterize the intracellular localization of functional CB_1 receptors. Previous reports suggested the association of CB_1 receptors with endolysosomal compartments (5, 6). The Golgi apparatus has also been involved in receptor internalization. To test which of these organelles are involved, we disrupted lysosomes with bafilomycin A_1 or the Golgi apparatus with brefeldin A. Pretreatment with bafilomycin A_1 markedly reduced the response to anandamide, whereas brefeldin A did not significantly affect it; these results indicate a critical role for acid-filled lysosomal Ca^{2+} stores in the anandamide-induced increase in $[Ca^{2+}]_i$ and suggest the localization of CB_1 receptors on endolysosomes. We also sought morphological evidence for localization of CB_1 receptors on lysosomes labeled with LAMP-1 and found that intracellular CB_1 receptors are indeed localized on lysosomes in CB_1-GFP-transfected HEK293 cells.

Next, we examined the presence of endoplasmic reticulum and lysosomal Ca^{2+} stores in CB_1 receptor-transfected

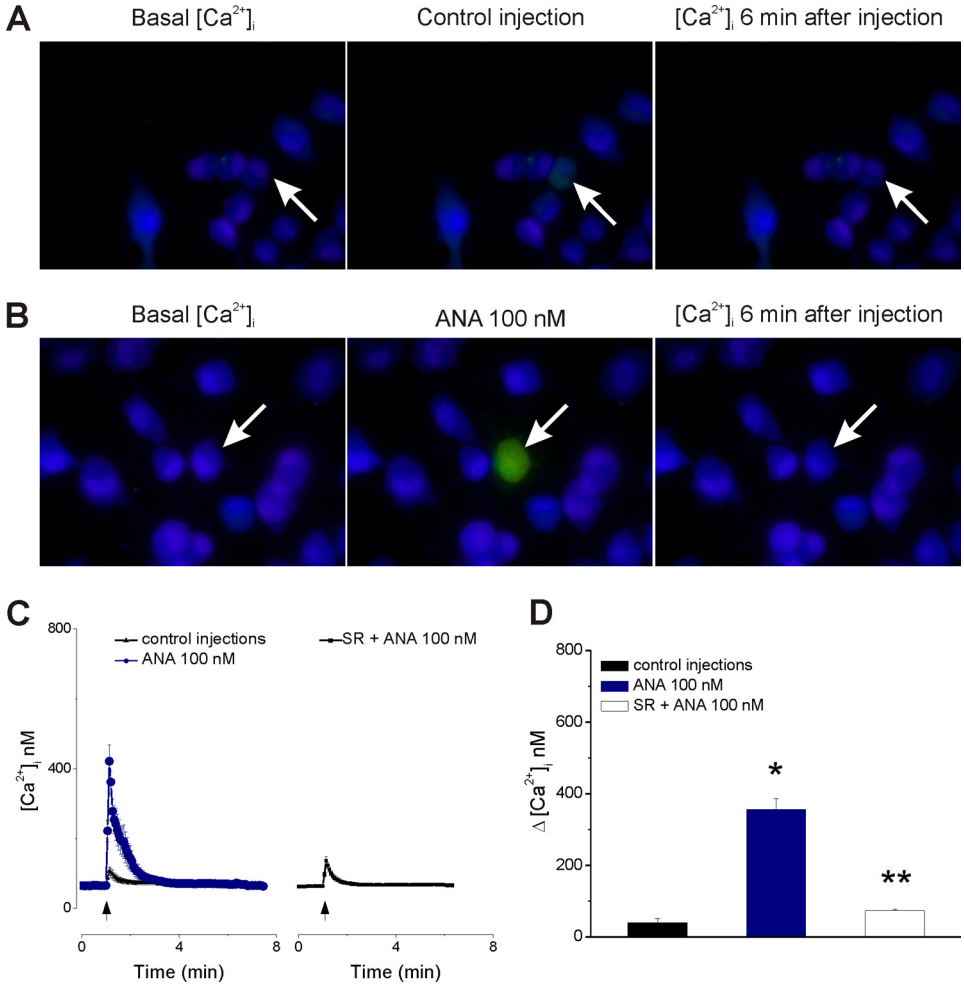

FIGURE 8. **Intracellular administration of anandamide increases $[Ca^{2+}]_i$ in NG108-15 cells.** *A* and *B*, fluorescent images of fura-2/AM-loaded-NG108-15 cells illustrating the levels of basal $[Ca^{2+}]_i$ (*left panels*), during control or anandamide (*ANA*; 100 nM) injection (*middle panels*), and 6 min after injection (*right panels*). Low levels of $[Ca^{2+}]_i$ are seen as *blue* fluorescence and higher levels as *green* and *red* fluorescence. The *arrows* indicate the injected cells. *C*, intracellular administration of anandamide (100 nM) produced a fast, robust, and transitory increase in $[Ca^{2+}]_i$. Averaged traces from six experiments are shown. Pretreatment with the CB_1 receptor antagonist SR141716A (*SR*) markedly reduced the response to anandamide. *D*, comparison of the increases in $[Ca^{2+}]_i$ produced by control injection (*black bar*) and anandamide (100 nM) in the absence (*dark blue column*) or presence (*white column*) of the antagonist. *, $p < 0.05$ compared with the control; **, $p < 0.05$ compared with anandamide (100 nM).

HEK293 cells. In Ca^{2+}-free Hanks' balanced salt solution supplemented with the Ca^{2+} chelator EGTA, thapsigargin (a sarcoplasmic/endoplasmic reticulum ATPase inhibitor) produced an increase in $[Ca^{2+}]_i$. Monensin, which collapses pH gradients across acidic organelles (34), added on the plateau of the thapsigargin-induced response produced a supplemental increase in $[Ca^{2+}]_i$. These results indicate that both endoplasmic reticulum and lysosomal Ca^{2+} stores are present in CB_1 receptor-transfected HEK293 cells.

The anandamide-induced $[Ca^{2+}]_i$ increase was not affected when experiments were carried out in Ca^{2+}-free saline, indicating that Ca^{2+} influx is not involved in this response. We further characterized the involvement of intracellular stores in the anandamide-induced $[Ca^{2+}]_i$ increase. NAADP, a potent Ca^{2+}-mobilizing second messenger, releases Ca^{2+} from acidic lysosome-like Ca^{2+} stores via recently identified two-pore channels (13, 35–37). Intracellular injection of NAADP elevated $[Ca^{2+}]_i$; the response was blocked by pretreatment with Ned-19, a specific inhibitor of NAADP signaling (28). IP_3 releases Ca^{2+} from the endoplasmic reticulum via IP_3 receptors, well characterized Ca^{2+} channels located on the endoplasmic reticulum (27). The increase in $[Ca^{2+}]_i$ produced by intracellular injection of IP_3 was blocked by xestospongin C and heparin, antagonists of IP_3 receptors.

Having established that lysosomes and the endoplasmic reticulum are not only present but also functional in CB_1 receptor-transfected HEK293 cells, we assessed their involvement in the Ca^{2+}-mobilizing effect of anandamide. We used two different approaches to assess the role of lysosomes. Besides disrupting the lysosomes with bafilomycin A1, we assessed also the effect of Ned-19, which blocks the NAADP-dependent lysosomal Ca^{2+} release without disrupting the lysosomes. Pretreatment with Ned-19 reduced the anandamide-induced Ca^{2+} response to a lesser extent than bafilomycin A_1. The difference in the magnitude of the Ca^{2+} responses to anandamide in the presence of bafilomycin A1 and Ned-19 reflects the different mechanisms employed by these two agents in interfering with the lysosomal Ca^{2+} release; it also indicates that intact lysosomes are important for the anandamide-induced Ca^{2+} responses. These results support the involvement of

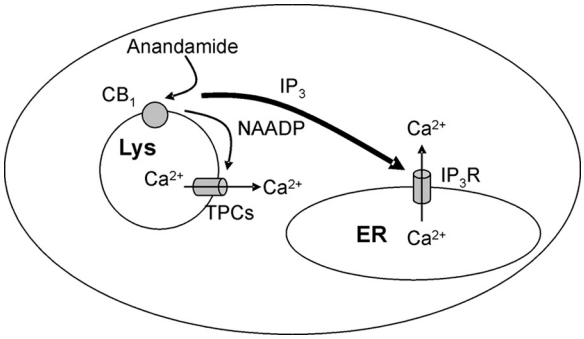

FIGURE 9. **Proposed mode of action of anandamide via intracellular CB_1 receptors.** Anandamide, acting on CB_1 receptors situated on acid-filled Ca^{2+} stores (endolysosomes), activates IP_3-dependent and NAADP-dependent Ca^{2+} pathways. IP_3 releases Ca^{2+} from the endoplasmic reticulum (*ER*) by activating the IP_3 receptor (*IP_3R*). NAADP, acting on recently identified two-pore channels (*TPCs*), releases Ca^{2+} from lysosomes (*Lys*).

NAADP-dependent lysosomal Ca^{2+} stores in the response to anandamide.

As earlier reports indicated that CB_1 receptor coupling to $G_{q/11}$ proteins may increase $[Ca^{2+}]_i$ (9), we tested whether the PLC-IP_3 pathway is involved in the effect of anandamide in CB_1 receptor-transfected HEK cells. Pretreatment with U73122, a PLC inhibitor (29), reduced but did not abolish the anandamide-induced increase in $[Ca^{2+}]_i$. The response was similar to that produced by anandamide in the presence of the IP_3 receptor antagonists xestospongin C and heparin and indicates the involvement of the PLC-IP_3 pathway, as reported previously (29). The amplitude of the increase in $[Ca^{2+}]_i$ produced by anandamide was higher than the sum of the NAADP-dependent and IP_3-dependent Ca^{2+} release, thus suggesting the participation of a Ca^{2+}-induced Ca^{2+} release mechanism in this response. Similarly, activation of NAADP receptors has been associated with the recruitment of inositol trisphosphate and/or ryanodine receptors via Ca^{2+}-induced Ca^{2+} release (38–43). However, because HEK293 cells lack ryanodine receptors (44), we suggest the involvement of the IP_3 receptor and a Ca^{2+}-induced Ca^{2+} release mechanism.

Several studies have revealed the presence of intracellular CB_1 receptors, including one showing functional CB_1 receptors in endolysosomes (reviewed in Ref. 45). Moreover, a recent study indicated that the intracellular pool of CB_1 receptors does not contribute to repopulation of membrane receptors (6), and thus, it may have a distinct function from the membrane CB_1 receptors.

Immunogold labeling studies have demonstrated the presence of CB_1 receptors intracellularly in axons of the basal ganglia (46) and locus coeruleus (47). Thus, if these intracellular receptors are active, they could play important roles in neuronal signaling. This is also supported by our novel finding that anandamide activates NAADP-dependent Ca^{2+} pathways. NAADP has been implicated in important neuronal functions, such as neurosecretion (48, 49), neurite outgrowth (42), neuronal differentiation (23), and membrane depolarization (50).

In sum, our findings indicate that intracellular CB_1 receptors are functional and that their activation mobilizes Ca^{2+} from the endoplasmic reticulum and lysosomes; the proposed model is summarized in Fig. 9. We have also reported, for the first time, a role for NAADP in cannabinoid-induced Ca^{2+} signaling.

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REFERENCES

1. McIntosh, H. H., Song, C., and Howlett, A. C. (1998) *Brain Res. Mol. Brain Res.* **53,** 163–173
2. Leterrier, C., Bonnard, D., Carrel, D., Rossier, J., and Lenkei, Z. (2004) *J. Biol. Chem.* **279,** 36013–36021
3. Leterrier, C., Lainé, J., Darmon, M., Boudin, H., Rossier, J., and Lenkei, Z. (2006) *J. Neurosci.* **26,** 3141–3153
4. McDonald, N. A., Henstridge, C. M., Connolly, C. N., and Irving, A. J. (2007) *Mol. Pharmacol.* **71,** 976–984
5. Rozenfeld, R., and Devi, L. A. (2008) *FASEB J.* **22,** 2311–2322
6. Grimsey, N. L., Graham, E. S., Dragunow, M., and Glass, M. (2010) *Biochem. Pharmacol.* **80,** 1050–1062
7. Felder, C. C., Briley, E. M., Axelrod, J., Simpson, J. T., Mackie, K., and Devane, W. A. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90,** 7656–7660
8. Felder, C. C., Veluz, J. S., Williams, H. L., Briley, E. M., and Matsuda, L. A. (1992) *Mol. Pharmacol.* **42,** 838–845
9. Lauckner, J. E., Hille, B., and Mackie, K. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102,** 19144–19149
10. Caulfield, M. P., and Brown, D. A. (1992) *Br. J. Pharmacol.* **106,** 231–232
11. Mackie, K., and Hille, B. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89,** 3825–3829
12. Tao, Q., and Abood, M. E. (1998) *J. Pharmacol. Exp. Ther.* **285,** 651–658
13. Brailoiu, E., Churamani, D., Cai, X., Schrlau, M. G., Brailoiu, G. C., Gao, X., Hooper, R., Boulware, M. J., Dun, N. J., Marchant, J. S., and Patel, S. (2009) *J. Cell Biol.* **186,** 201–209
14. Brailoiu, E., Hooper, R., Cai, X., Brailoiu, G. C., Keebler, M. V., Dun, N. J., Marchant, J. S., and Patel, S. (2010) *J. Biol. Chem.* **285,** 2897–2901
15. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450
16. Eskelinen, E. L., Tanaka, Y., and Saftig, P. (2003) *Trends Cell Biol.* **13,** 137–145
17. Ho, B. Y., and Zhao, J. (1996) *Neurosci. Lett.* **212,** 123–126
18. Schachter, J. B., Sromek, S. M., Nicholas, R. A., and Harden, T. K. (1997) *Neuropharmacology* **36,** 1181–1187
19. Rinaldi-Carmona, M., Barth, F., Héaulme, M., Shire, D., Calandra, B., Congy, C., Martinez, S., Maruani, J., Néliat, G., and Caput, D. (1994) *FEBS Lett.* **350,** 240–244
20. Bowman, E. J., Siebers, A., and Altendorf, K. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85,** 7972–7976
21. Churchill, G. C., Okada, Y., Thomas, J. M., Genazzani, A. A., Patel, S., and Galione, A. (2002) *Cell* **111,** 703–708
22. Kinnear, N. P., Boittin, F. X., Thomas, J. M., Galione, A., and Evans, A. M. (2004) *J. Biol. Chem.* **279,** 54319–54326
23. Brailoiu, E., Churamani, D., Pandey, V., Brailoiu, G. C., Tuluc, F., Patel, S., and Dun, N. J. (2006) *J. Biol. Chem.* **281,** 15923–15928
24. Zhang, F., Zhang, G., Zhang, A. Y., Koeberl, M. J., Wallander, E., and Li, P. L. (2006) *Am. J. Physiol. Heart Circ. Physiol.* **291,** H274–H282
25. Zhang, F., Xia, M., and Li, P. L. (2010) *Am. J. Physiol. Cell Physiol.* **298,** C1209–C1216
26. Lee, H. C., and Aarhus, R. (1995) *J. Biol. Chem.* **270,** 2152–2157
27. Berridge, M. J. (2002) *Cell Calcium* **32,** 235–249
28. Naylor, E., Arredouani, A., Vasudevan, S. R., Lewis, A. M., Parkesh, R., Mizote, A., Rosen, D., Thomas, J. M., Izumi, M., Ganesan, A., Galione, A., and Churchill, G. C. (2009) *Nat. Chem. Biol.* **5,** 220–226
29. Bleasdale, J. E., Thakur, N. R., Gremban, R. S., Bundy, G. L., Fitzpatrick,

F. A., Smith, R. J., and Bunting, S. (1990) *J. Pharmacol. Exp. Ther.* **255,** 756–768
30. Di Marzo, V. (2008) *Nat. Rev. Drug Discov.* **7,** 438–455
31. Piomelli, D. (2003) *Nat. Rev. Neurosci.* **4,** 873–884
32. Brailoiu, E., Filipeanu, C. M., Tica, A., Toma, C. P., de Zeeuw, D., and Nelemans, S. A. (1999) *Br. J. Pharmacol.* **126,** 1133–1138
33. Re, R. N., and Cook, J. L. (2007) *Nat. Clin. Pract. Cardiovasc. Med.* **4,** 549–557
34. Styrt, B., Schwartz, M. A., and Klempner, M. S. (1988) *Biochem. Biophys. Res. Commun.* **154,** 1308–1313
35. Calcraft, P. J., Ruas, M., Pan, Z., Cheng, X., Arredouani, A., Hao, X., Tang, J., Rietdorf, K., Teboul, L., Chuang, K. T., Lin, P., Xiao, R., Wang, C., Zhu, Y., Lin, Y., Wyatt, C. N., Parrington, J., Ma, J., Evans, A. M., Galione, A., and Zhu, M. X. (2009) *Nature* **459,** 596–600
36. Galione, A., Evans, A. M., Ma, J., Parrington, J., Arredouani, A., Cheng, X., and Zhu, M. X. (2009) *Pflugers Arch.* **458,** 869–876
37. Patel, S., Marchant, J. S., and Brailoiu, E. (2010) *Cell Calcium* **47,** 480–490
38. Cancela, J. M., Churchill, G. C., and Galione, A. (1999) *Nature* **398,** 74–76
39. Santella, L., Kyozuka, K., Genazzani, A. A., De Riso, L., and Carafoli, E. (2000) *J. Biol. Chem.* **275,** 8301–8306
40. Churchill, G. C., and Galione, A. (2001) *EMBO J.* **20,** 2666–2671
41. Boittin, F. X., Galione, A., and Evans, A. M. (2002) *Circ. Res.* **91,** 1168–1175
42. Brailoiu, E., Hoard, J. L., Filipeanu, C. M., Brailoiu, G. C., Dun, S. L., Patel, S., and Dun, N. J. (2005) *J. Biol. Chem.* **280,** 5646–5650
43. Macgregor, A., Yamasaki, M., Rakovic, S., Sanders, L., Parkesh, R., Churchill, G. C., Galione, A., and Terrar, D. A. (2007) *J. Biol. Chem.* **282,** 15302–15311
44. Aoyama, M., Yamada, A., Wang, J., Ohya, S., Furuzono, S., Goto, T., Hotta, S., Ito, Y., Matsubara, T., Shimokata, K., Chen, S. R., Imaizumi, Y., and Nakayama, S. (2004) *J. Cell Sci.* **117,** 2813–2825
45. Rozenfeld, R. (2011) *Traffic* **12,** 12–18
46. Pickel, V. M., Chan, J., Kash, T. L., Rodríguez, J. J., and MacKie, K. (2004) *Neuroscience* **127,** 101–112
47. Scavone, J. L., Mackie, K., and Van Bockstaele, E. J. (2010) *Brain Res.* **1312,** 18–31
48. Brailoiu, E., Miyamoto, M. D., and Dun, N. J. (2001) *Mol. Pharmacol.* **60,** 718–724
49. Chameau, P., Van de Vrede, Y., Fossier, P., and Baux, G. (2001) *Pflugers Arch.* **443,** 289–296
50. Brailoiu, G. C., Brailoiu, E., Parkesh, R., Galione, A., Churchill, G. C., Patel, S., and Dun, N. J. (2009) *Biochem. J.* **419,** 91–97